IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Chenicheri Hariharan Nair et al.

Serial No.: 10/029,026 Filed: December 21, 2001

Examiner: Not Yet Known Group Art Unit: 1741

For: APPARATUS AND METHOD FOR SEPARATION OF MOLECULES AND MOVEMENT OF FLUIDS

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Frank M. Gasparo, Esq. (Reg. No. 44,700) March 18, 2002 Date

Commissioner for Patents
Washington, D.C. 20231

SUBMISSION OF PRIORITY DOCUMENT

SIR:

Applicants hereby submit a certified copy of the priority document: Australian Provisional Application No. PR 2224 filed on December 21, 2000 in the name of Gradipore Limited.

The Commissioner for Patents is hereby authorized to charge payment of all fees associated with this communication to Deposit Account No. 02-0393.

Respectfully submitted,

Date: March 18, 2002

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Enclosure

AUSTRALIA

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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 2224 for a patent by GRADIPORE LIMITED filed on 21 December 2000.

WITNESS my hand this
Thirteenth day of December 2001

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

Gradipore Limited

PROVISIONAL SPECIFICATION

Invention Title:

Apparatus and method for separation of molecules and movement of fluids

The invention is described in the following statement:

Technical Field

The present invention relates to method and apparatus for the separation of compounds, particularly macromolecules, where movement of fluids can be managed.

Background Art

Gradiflow™ is a new technology originally developed for the separation of macromolecules such as proteins, nucleotides and complex sugars. The Gradiflow™ process provides a high purity, scalable separation that is faster, cheaper and higher yielding than current methods of macromolecule separation and offers the potential to concurrently purify and detoxify macromolecule solutions.

Gradiflow™ is a unique preparative electrophoresis technology for macromolecule separation which utilises tangential flow across a polyacrylamide membrane when an electric field or potential is applied across the membrane (AU 601040). The general design of the Gradiflow™ system facilitates the purification of proteins and other macromolecules under near native conditions. This results in higher yields and excellent recovery. Some examples of Gradiflow™ technology may be found in US Patent Numbers 5039386 and 5650055, which US Patents are incorporated herein by reference.

In essence the Gradiflow™ technology is bundled into a cartridge comprising a number of membranes housed in a system which allows separation of macromolecules by charge and/or molecular weight. The system can also concentrate and desalt/dialyse at the same time. The multimodal nature of the system allows this technology to be used in a number of other areas especially in the production of biological components for medical use. The structure of the membranes may be configured so that biological contaminants can also be removed at the point of separation - a task which is not currently available in the biotechnology industry and which adds to the cost of production through time delays and due to the complexity of the task.

Gradiflow™ technology provides a new, potentially viable alternative to conventional chromatography separation techniques. The resolution of preparative electrophoresis and the scalable nature of Gradiflow™ technology point to it as being the future of biopharmaceutical production.

The transfer of fluid from on area to another, through a porous membrane, under electrophoretic conditions, is called electro-endo-osmosis. Electro-endo-osmosis is a natural occurrence with Gradiflow™ technology and its management can result in the increase of product recovery, decrease in run times and concentrate samples. These improvements can be achieved by maintaining the concentration of the target molecule, in a specific steam, by managing the extent of bulk fluid transfer. One method to manage electro-endo-osmosis was via electro-osmosis, were an external power source alters the rate of a system undergoing osmosis or endo-osmosis. The impact of large volume increase is potentially more serious in the scale-up use of the Gradiflow™ system. Control of electro-endo-osmosis would contribute significantly to cost reduction and efficiencies in plant maintenance.

The present inventors have now developed a modification of the Gradiflow™ technology to assist in the management of bulk fluid transfer/endo-osmosis.

Disclosure of Invention

In a first aspect, the present invention provides an electrophoresis apparatus, the apparatus comprising:-

(a) an upper buffer chamber and a lower buffer chamber, each chamber having inlet and outlet means for feeding liquid into and out of the chambers;

(b) a sample chamber and a separation chamber positioned between the upper and lower buffer chambers, the sample and separation chambers being formed by a plurality of membranes positioned between the upper and lower buffer chambers, at least one of the membranes being a barrier capable of preventing the substantial bulk movement of fluid under the influence of an electric field; and

(c) inlet and outlet means for feeding liquid into and out of the sample and separation chambers.

Preferably, the sample and separation chambers are formed or housed in a cartridge which is adapted to be removable from the apparatus.

Preferably, the barrier is an inducible electro-endo-osmotic membrane.

In another preferred form, some of the membranes are electrophoresis separation membranes and others are restriction membranes. Preferably, the restriction membranes are positioned between the upper or lower buffer chambers and the adjacent separation or sample chamber. At least one of the

restriction membranes is the inducible electro-endo-osmotic membrane which prevents the substantial bulk movement of fluid under the influence of an electric field.

The electrophoresis separation membranes are preferably made from polyacrylamide and have a molecular mass cut-off of at least about 3 kDa. The molecular mass cut-off of the membrane will depend on the sample being processed, the other molecules in the sample mixture, and the type of separation carried out.

One of restriction membrane is also preferably formed from polyacrylamide. The molecular mass cut-off of the restriction membrane will depend on the sample being processed, the other molecules in the sample mixture, and the type of separation carried out.

The inducible electro-endo-osmotic membrane is preferably a cellulose tri-acetate (CTA) membrane. It will be appreciated that the inducible electro-endo-osmotic membrane can be formed from any other suitable membrane material such as poly(vinyl alcohol) crosslinked with glutaraldehyde (PVAl+glut.

The present inventors have found that a CTA having a nominal molecular mass cut-off of 5, 10 or 20 kDa is particularly suitable for use in the apparatus.

In a preferred from, the apparatus further comprises:-

(d) electrodes housed in the upper and lower buffer chambers.

In one particularly preferred embodiment, the electrodes are made of titanium mesh coated with platinum.

The upper and lower electrodes may be housed in recesses or channels defined in upper and lower connection blocks, respectively. Those channels may define part of the boundaries of the buffer flow path for the apparatus. The other boundary for the buffer flow path is defined by the restriction/inducible electro-endo-osmotic membranes housed in the cartridge which form the upper and lower faces of the cartridge. The upper and lower connection blocks may also define inlets and outlets for buffer flow.

In a related aspect, the invention also encompasses a cartridge for use in the apparatus of the present invention, the cartridge including a housing and containing a separation membrane, a flow path defined along one side of the separation membrane being the sample chamber, and a flow path defined along an opposite side of the separation membrane being the separation

chamber, and restriction/inducible electro-endo-osmotic membranes for isolating buffer flow from sample and separation chambers, respectively.

The cartridge may further include gaskets positioned either side of the restriction membranes for sealing the components between the upper and lower connection blocks. The flow paths on each side of the membranes may be defined by grid elements. Each grid element may be generally planar. The grid element and restriction membranes may include a through hole located near each end of the element/membrane for the passage of liquid therethrough to the separation chamber. The grid elements may include a central elongate at least part cut out portion which in the assembled cartridge in conjunction with the two adjacent membranes, defines a flow path or channel from the hole one end of the grid element to the hole at the other end. The flow paths may include a lattice for supporting the separation membrane and for mixing the flow.

All the elements of the cartridge apart form the cartridge housing or casing are generally planar and are assembled together in a sandwich type construction.

It will be appreciated that the cartridge can define multiple sample and separation chambers by having a number of separation membranes positioned between a number of restriction/inducible electro-endo-osmotic membranes. This allows several separations, from the same of different starting samples, to be carried out simultaneously.

In use, buffer is circulated through the upper and lower buffer chambers and liquid sample is applied to the sample chamber through the inlet and outlet means. When an electric potential or field is applied to the apparatus via the electrodes, some components in the sample will be caused to move through the membrane into the adjacent separation chamber or a buffer chamber. It is possible to retrieve the component moved into the sample chamber through its inlet or outlet means. The inducible electro-endo-osmotic membrane prevents substantial bulk fluid movement between the chambers thereby preventing undesirable dilution of the sample or separated compound during the separation process.

The distance between the electrodes can have an effect on the separation or movement of compounds through the membranes. It has been found that the shorter the distance between the electrodes, the faster the electrophoretic movement of compounds.

The membranes may be formed as a multilayer or sandwich arrangement. The thickness of the membranes can have an effect on the separation or movement of compounds. It has been found that the thinner the membrane, faster and more efficient movement occurs.

The restriction/inducible electro-endo-osmotic membrane positioned between the upper buffer stream and the sample chamber and between the separation chamber adjacent the lower buffer stream can have the same molecular mass cut-off or different cut-offs therefore forming an asymmetrical arrangement.

Flow rates of buffer and of the sample through the buffer and separation chambers can have an influence on the separation of compounds. Rates of milliliters per minute up to liters per minute can be used depending on the configuration of the apparatus and the sample to be separated.

The temperature of buffers and sample solutions in the apparatus can be controlled by a suitable cooling/heating means. The apparatus may also be positioned in a controlled-temperature environment to maintain a desired temperature during use.

The system may have its own power supply or can be connected to an external power supply.

Voltage and/or current applied can vary depending on the separation. Typically up to about 500 volts can used but choice of voltage will depend on the configuration of the apparatus, buffers and the sample to be separated.

In a second aspect, the present invention provides a method for separating at least one component from a sample while controlling the bulk movement of fluid, the method comprising the steps of:-

(a) providing an electrophoresis apparatus having an upper buffer chamber and a lower buffer chamber, each chamber having inlet and outlet means for feeding liquid into and out of the buffer chamber; a sample chamber and a separation chamber positioned between the upper and lower buffer chambers, the sample and separation chambers being formed by a plurality of membranes positioned between the upper and lower buffer chambers, at least one of the membranes being an inducible electro-endo-osmotic membrane capable of preventing the substantial bulk movement of fluid under the influence of an electric field; and inlet and outlet means for feeding liquid into and out of the sample and separation chambers;

(b) adding buffer to the upper and lower buffer chambers;

(c) adding the sample to the sample chamber via the means for introducing or removing liquid of the chamber;

(d) adding buffer or fluid to the separation chamber;

(e) applying an electric potential between the electrodes causing at least one compound form the sample in the sample chamber to move through the membrane into the adjacent separation chamber; and optionally

(f) collecting the separated compound;

wherein in use the inducible electro-endo-osmotic membrane limits the bulk movement of fluid into the sample or separation chambers.

In one preferred form, two apparatus are connected in a manner so as to further control or prevent bulk movement of fluid into the sample or separation chambers. The first apparatus functions to separate the compound of choice while the second apparatus allows the concentration of the separated compound. Preferably, the electric potential applied to each apparatus is under separate control. In a preferred form, the separation chamber of the first apparatus is in fluid connection with the sample chamber of the second apparatus and the sample chamber of the first apparatus is in fluid connection with the separation chamber of the second apparatus. In use, after the compound to be separated is caused to move in the separation chamber of the first apparatus, it is then transferred to the sample chamber of the second apparatus which causes unwanted fluid so move to the separation chamber which is then passed to the sample chamber of the first apparatus. This dual apparatus system functions as further a sample concentrator without the total loss of fluid or sample.

In a third aspect, the present invention relates to use of the apparatus according to the first aspect of the present invention in the separation of one or more compounds from a sample.

In a fourth aspect, the present invention relates to use of the method according to the second aspect of the present invention in the separation of one or more compounds from a sample.

Gradiflow™ is a trade mark owned by Gradipore Limited, Australia.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any description of prior art documents herein is not an admission that the documents form part of the common general knowledge of the relevant art in Australia.

In order that the present invention may be more clearly understood preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1 shows PAGE of CTA calibration experiments.

Figure 2 shows endo-osmosis rates with CTA membranes.

Figure 3 shows comparison of CTA orientation and the endo-osmotic rate.

Figure 4 shows rate of volume removal due to electro-endo-osmosis.

Figure 5 shows BSA recovery with voltage change.

Figure 6 shows rate of volume removal due to electro-endo-osmosis.

Figure 7 shows a preferred apparatus plumbing for Method 3, which incorporates an upstream concentrator machine for the management of endo-osmosis.

Figure 8 shows comparison of endo-osmotic rate and percentage fibrinogen recovered.

Modes for Carrying Out the Invention

INVESTIGATIONS

The following were investigated:

a) The molecular weight cut-off of cellulose tri-acetate (CTA) under electrophoretic conditions.

b) The orientation of CTA membranes.

c) Whether CTA endo-osmosis is voltage dependent.

d) Whether CTA endo-osmosis is pH dependent.

e) Can CTA be incorporated into Gradiflow technology to improve yield, purification rates and/or act as a concentrator.

EXPERIMENTATION AND RESULTS

Materials

Gradiflow™ machine: BF100 and BF200

5 kDa, 500 kDa, 700 kDa and 1500 kDa molecular mass cut-off polyacrylamide membranes (Gradipore Limited)

5 kDa, 10 kDa and 20 kDa molecular mass cut-off CTA membranes (Sartorius)

BSA 67 kDa pI~5, ovalbumin 45 kDa pI 4.2, trypsin inhibitor 14 kDa pI 4.7, and fibrinogen 330 kDa pI 5.5

Calibration of the molecular mass cut-off of CTA membranes.

The molecular mass cut-off for the 10 kDa and 20 kDa CTA membranes were determined for use in Gradiflow™ technology. The 10 kDa and 20 kDa CTA membranes were used in separate purification runs were 3 mg/mL protein mixture (1 mg/mL of each - BSA, ovalbumin and trypsin inhibitor in Tris Buffer (TB) pH 8.0) was placed in the upstream and separated with 250 V for 45 min into the downstream. A non-reduced PAGE of the 0 min upstream and 45 min downstream was run to determine the molecular mass cut-off of the 10 kDa and 20 kDa CTA membranes (Figure 1).

The results of the CTA calibration experiments (Figure 1), suggest that under electrophoretic conditions the actual cut-offs for the CTA membranes are 14 kDa for the 10 kDa CTA and 45 kDa for the 20 kDa CTA membranes. The 14 kDa molecular mass cut off of the 10 kDa CTA membrane was determined from the PAGE as only trypsin inhibitor (14 kDa) was observed after passing through the 10 kDa CTA membrane during the 45 min separation run. The 45 kDa molecular mass cut-off of the 20 kDa CTA membrane was determined from the PAGE as trypsin inhibitor (14 kDa), ovalbumin (45 kDa) and a small quantity of BSA (67 kDa) passed through the 20 kDa CTA membrane during the 45 min separation run.

Orientation of CTA membranes

CTA membranes are asymmetrical, therefore to use CTA membranes for further experimentation, the orientation (shiny side up or down) and endo-osmotic rate of 5 kDa, 10 kDa and 20 kDa CTA membranes was analysed. These parameters were investigated in two-stream and single-stream configuration, in order to identify which configuration had the highest

endo-osmotic rate. This involved recording volume variations every 5 min and comparing the amount of starting BSA (1 mg/mL) to the final amount of BSA.

The experimental data suggested that the 5 kDa CTA membrane had the slowest endo-osmotic rate in both the standard (twin) and single-steam configurations, compared to the other CTA membranes (Figure 2). The endo-osmotic rates for both the 10 kDa and 20 kDa membranes were comparably high, with the single stream configuration demonstrating a higher endo-osmotic rate than the standard configuration (Figure 2). The 10 kDa CTA membrane had the highest endo-osmotic rate in both configurations compared to the 5 kDa and 20 kDa CTA membranes.

As the CTA membranes are asymmetrical (a shiny side and a dull side) a comparison of the orientation was also conducted. The results indicated that the endo-osmotic rate of the shiny side up 5 kDa CTA membrane was higher than the shiny side down (Figure 3). However, the 10 kDa and 20 kDa shiny side down CTA membranes performed slightly better than the shiny side up configuration. Overall, the 10 kDa CTA membrane had a higher endo-osmotic rate.

In conclusion, single stream configuration Gradiflow™ technology using a 10 kDa CTA membrane, in either symmetry, produced the highest endo-osmotic rate. This provided the foundation for the further experimental procedures. For consistency, all remaining experiments were single-stream configuration, using a 10 kDa shiny side up CTA membrane.

Effect of Voltage on Endo-Osmosis

To determine whether endo-osmosis was voltage dependent, a series of experiments in which the volume loss due to electro-osmosis was prepared. To investigate whether CTA endo-osmosis was voltage dependent, a Gradiflow™ apparatus was set-up in single-stream configuration, with cooled TB pH 9.0 in the buffer tank, and a cartridge consisting of a lower polyacrylamide restriction membrane and an upper CTA separating membrane. Fifty mL of 1 mg/mL BSA was run through the cartridge in the downstream of the Gradiflow™ machine for 30 min. The change in volume was measured every 5 min and the concentration of the initial and final downstream were determined by spectrophotometry (A_{280}). The above

experimental set-up was repeated from 0 V to 300 V (1A, 300W) at 50 V increments.

The experimental data shows that as the voltage increased, the rate of volume loss increased linearly (Figure 4). The results suggest that the endo-osmotic rate, in conjunction with CTA as a restriction membrane, can be managed by altering the voltage for procedures in which bulk fluid transfer is an issue. However, as the voltage increased the amount of BSA recovered was reduced (Figure 5). The reduced recovery of BSA was attributed to the increased voltage forcing the BSA into the restriction membrane.

Effect of pH on Endo-Osmosis

To determine whether endo-osmosis was pH dependent, a series of experiments in which the volume loss due to electro-endo-osmosis were prepared. To investigate whether CTA endo-osmosis was pH dependent, a Gradiflow™ apparatus was set-up in single-stream configuration, with cooled TB pH 9.0 in the buffer tank, and a cartridge consisting of a bottom polyacrylamide restriction membrane and a top CTA separating membrane. Fifty mL of 1 mg/mL BSA was run through the cartridge in the downstream of the apparatus for 30 min at 250 V (1A, 300W). The change in volume was measured every 5 min and the concentration of the initial and final downstream were determined by spectrophotometry (A_{280}). The above experimental set-up was repeated from pH 4.0 to pH 9.0, at pH 0.5 increments.

The experimental data suggests that as the pH increased, the rate of volume loss increases (Figure 6). However, each buffer had its own variations, suggesting that pH dependent endo-osmosis would need to be investigated on an individual project/buffer basis.

Endo-Osmosis Management and the Isolation of Fibrinogen

To test whether the endo-osmotic rate could be managed with a inducible electro-endo-osmotic membrane, a procedure with a high endo-osmotic rate was studied. The procedure with a high endo-osmotic rate was the isolation of fibrinogen from cryo-precipitate. Isolating fibrinogen from cryo-precipitate typically results in the increase of the upstream volume by a factor of 5 to 8 from the downstream. In order to alter the upstream endo-osmosis rate, fibrinogen was isolated from a stock cryo-precipitate (1:3

dilution cryo-precipitate stock solution was prepared and used as the starting material for all three methods) using three different methods:

Method 1:

The first method was the standard isolation of fibrinogen using the Gradiflow™ with a 700-1500-700 kDa cartridge configuration at 250 V (1A, 300W). The buffer tank contained cooled TB pH 9.0, the upstream 30 mL 1:3 diluted cryo-precipitate stock solution and 10 mL TB pH 9.0 in the downstream. The change in upstream volume was noted every 15 min, along with a spectrophotometry (OD_{280}) reading of the downstream. The downstream was harvested every 30 min, the buffer replaced and the voltage reversed for 2 min (to de-foul the membrane).

Method 2:

The second method was similar to Method 1, with the exception that the top restriction membrane (700 kDa polyacrylamide) was replaced with a 10 kDa CTA membrane.

Method 3:

The third method used two Gradiflow™ apparatus, with separate power supplies, attached together (Figure 7). Apparatus 1 was prepared and run as described in Method 1 (note: the 700 kDa restriction membranes were replaced with 500 kDa restriction membranes), apparatus 2, however, was configured as a single-stream with a 5 kDa polyacrylamide (bottom) -10 kDa CTA (top). Apparatus 2 was connected to the upstream of apparatus 1 and functioned as an upstream concentrator, by managing the endo-osmotic rate with the separate power supply. However, from the data obtained from the voltage dependence experiments, a voltage which matched the endo-osmotic rate of Method 1 was chosen. The voltage of apparatus 2 was 250 V (1A, 300W).

Using CTA membrane during fibrinogen isolation was found to decrease the amount of endo-osmosis (Figure 8) by a factor of 2 (Method 2) to 6 (Method 3). Using a separate power supply and Gradiflow™ with a concentrator cartridge, the upstream endo-osmosis rate was maintained at a low endo-osmotic rate, without altering voltage (voltage was set at a constant 250 V). Unlike Method 3, the downstream volume of Methods 1 and 2 required continual monitoring and toping up.

Method 1 isolated 34.8% fibrinogen, while Method 2 isolated 42.6% fibrinogen and Method 3 isolated 53.5% fibrinogen from a stock cryo-

precipitate solution (Figure 8). Overall, Methods 2 and 3 appeared to be commercially suitable as they resulted in a 9-20% increase in fibrinogen recovered and a 44-94% decrease of the endo-osmosis rate.

CONCLUSION

The highest endo-osmotic rate was produced using a single-stream configuration Gradiflow™ with a 10 kDa CTA membrane, in either symmetry. The identification of the configuration with the highest endo-osmotic rate allowed for experiments to manage this rate. Analysing whether endo-osmosis was voltage dependent demonstrated that the rate of volume loss linearly increased as the voltage increased. Therefore, increasing the voltage of a system can reduce the effect of endo-osmosis by electro-osmosis. The relationship between pH and endo-osmosis suggests that each buffer system had it own variables and that pH would need to be investigated on an individual project basis.

To test whether the endo-osmotic rate could be managed with a inducible electro-endo-osmotic membrane (CTA), a procedure with a high endo-osmotic rate was studied. The procedure with a high endo-osmotic rate was the isolation of fibrinogen from cryo-precipitate. Three experiments were used to study endo-osmosis management. The first experiment was a control which identified the endo-osmosis rate of a standard fibrinogen isolation run. The second experiment involved reducing the endo-osmotic rate by replacing the top polyacrylamide restriction membrane with a CTA membrane. The final experiment utilised a second apparatus, in a single-stream configuration (CTA top and polyacrylamide on the bottom), which managed the upstream volume of the first apparatus. Each apparatus had its own power supply so that the rate of electro-osmosis in apparatus 2 could be managed by varying the voltage (matching the electro-osmotic rate). The results of these experiments demonstrated that endo-osmotic rate of the upstream could be managed thereby increasing total recovery of fibrinogen. The increased fibrinogen recovery rate was achieved by maintaining the concentration of the target molecule, in a specific steam, by managing the extent of bulk fluid transfer. The bulk fluid transfer was managed with Gradiflow™ technology, a inducible electro-endo-osmotic membrane and a potential.

The endo-osmotic rate was managed using Gradiflow™ technology in conjunction with a inducible electro-endo-osmotic membrane and variable

voltage, which induced electro-endo-osmosis. Managing the electro-endo-osmotic rate can increase product recovery, decrease run times and concentrate samples.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty-first day of December 2000

Gradipore Limited
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1 PAGE of CTA calibration experiments

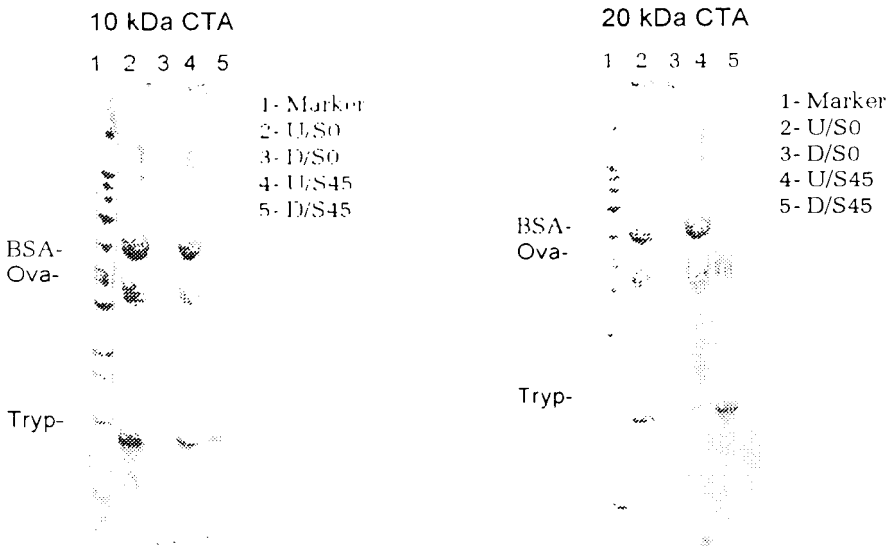


Figure 2 Endo-Osmosis Rates with CTA Membranes.

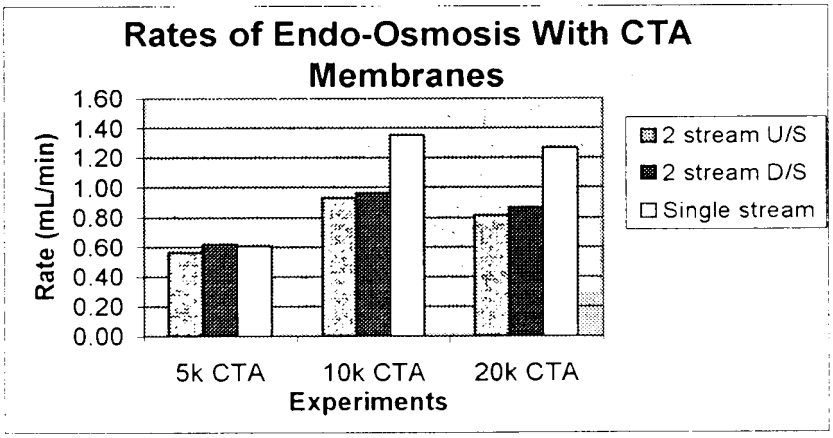


Figure 3 Comparison of CTA Orientation and the Endo-Osmotic Rate.

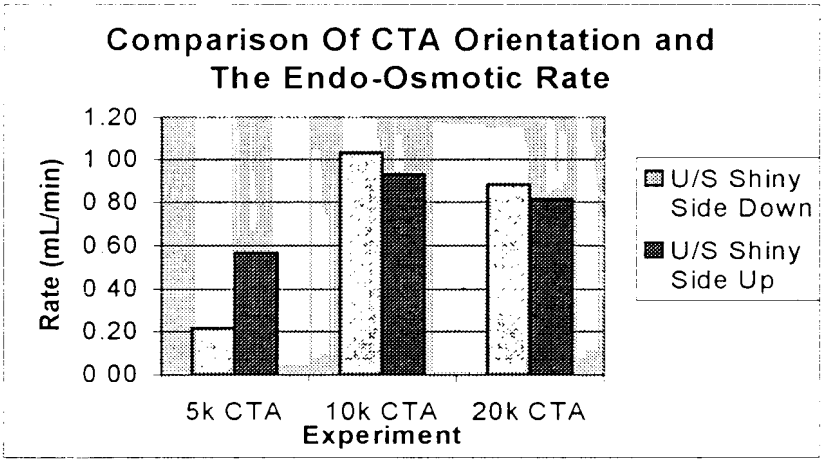


Figure 4 Rate of volume removal due to electro-endo-osmosis.

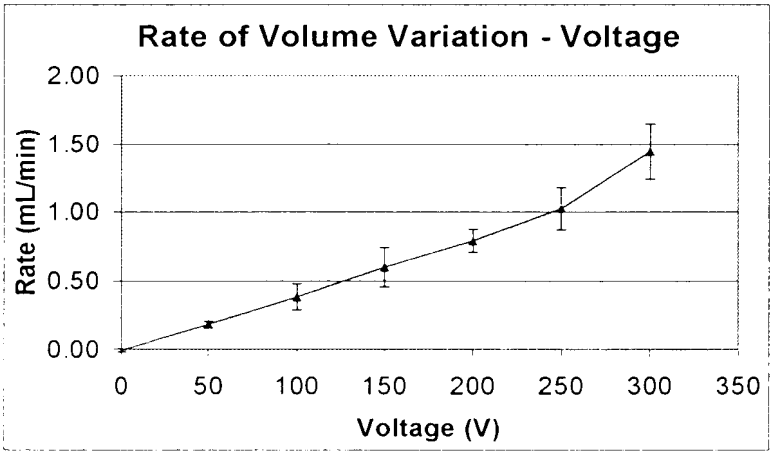


Figure 5 BSA recovery with voltage change.

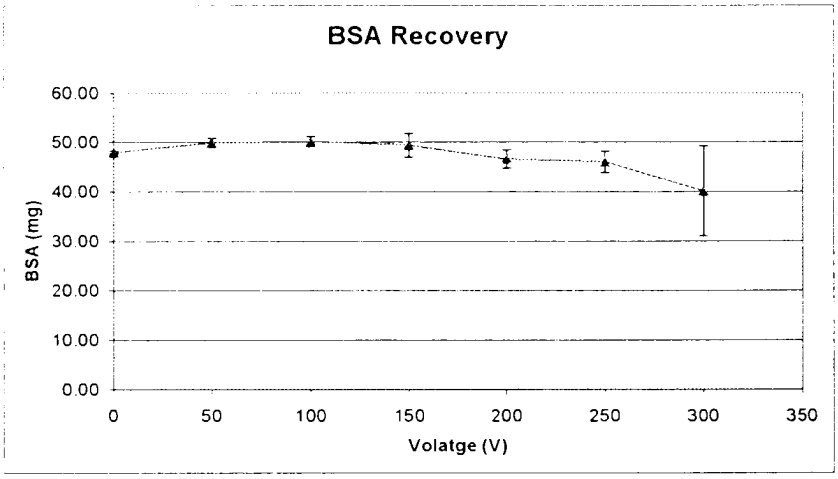
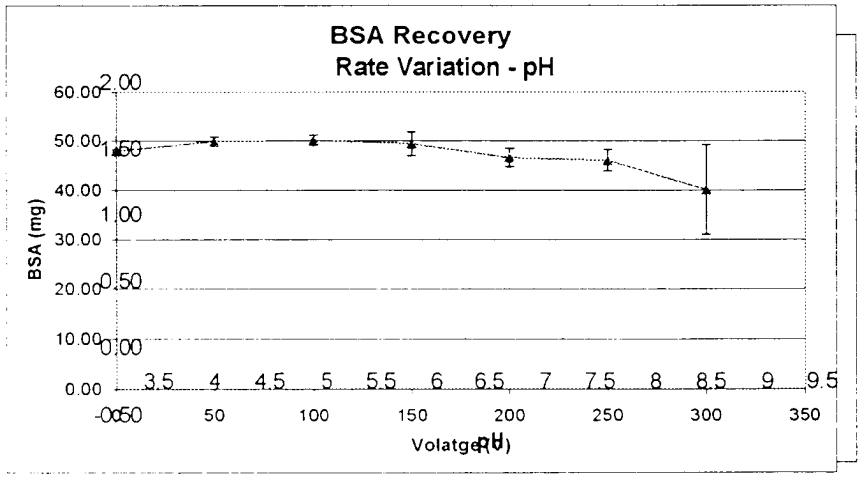


Figure 6 Rate of volume removal due to electo-endo-osmosis.



Buffer Used

acetic acid/GABA

MES/histidine

Figure 7 The plumbing for Method 3, which incorporation an upstream concentrator machine for the management of endo-osmosis.

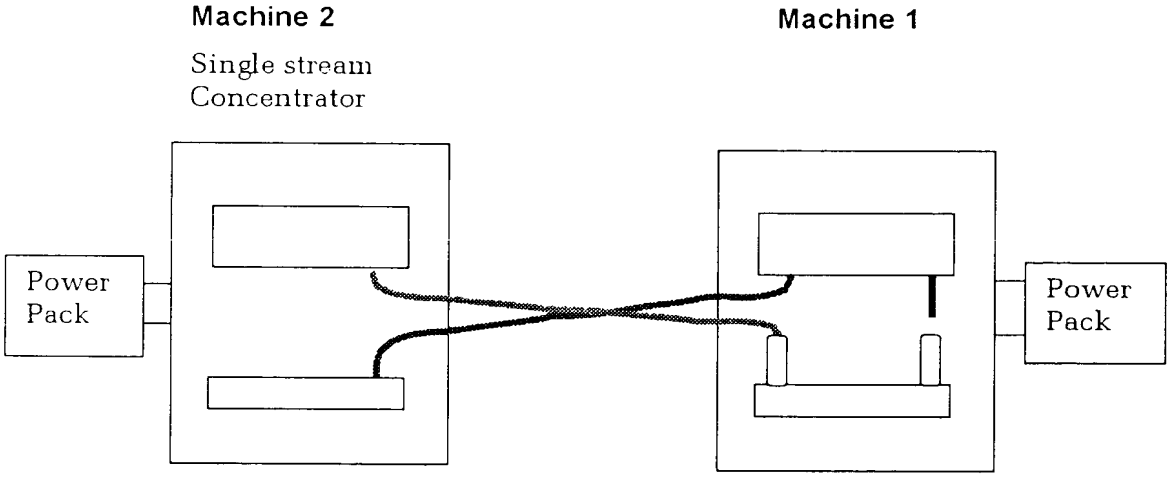


Figure 8 Comparison of Endo-Osmotic Rate and percentage Fibrinogen recovered.